DIAGNOSIS OF PLASMODIUM INFECTION BY ANALYSIS OF EXTRACHROMOSOMAL GENETIC MATERIAL

FIELD OF THE INVENTION

The present invention relates generally to novel genetic sequences isolated from a parasitic protozoa which infects humans and other animals and the uses of said sequences as diagnostic agents for the detection of said protozoa in a biological sample. In particular, the present invention provides genetic sequences of the extrachromosomal genetic elements of the malaria agents *Plasmodium berghei*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium falciparum* and *Plasmodium malariae* and synthetic oligonucleotide derivatives, homologues, analogues and fragments thereof. The genetic sequences of the present invention are particularly useful in the diagnosis, prophylactic treatment and therapeutic treatment of humans and other animals which are capable of being infected by or are actually infected by protozoa such as *Plasmodium ssp.*, for example *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, *P. cynomolgi*, *P. gonderi*, *P. (Hepatocytis) kochi*, *P. inui*, *P. knowlesi*, *P. reichenowi*, *P. rodhaini*, *P. schwetzi*, *P. cathemerium*, *P. elongatum*, *P. relictum*, *P. lophurae*, *P. gallinaceum*, *P.chabaudi*, *P. yoelii*, or *P. berghei*, amongst others. The invention provides further, a novel, reliable diagnostic assay for the detection of *Plasmodium ssp.* in humans and animals.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description. Sequence identity numbers (SEQ ID Nos.) for the nucleotide and amino acid sequences referred to in the specification are defined after the bibliography.

Throughout the specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers, but not the exclusion of any other element or integer or group of elements or integers.

BACKGROUND TO THE INVENTION

More than fifty different species of *Plasmodium* can cause malaria in humans, monkeys,

birds, fish, cattle and rodents. The development of diagnostic assays for the detection of *Plasmodium* in humans and animals is therefore highly desirable.

Human malaria, which is caused by *Plasmodium ssp.*, in particular *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, remains one of the major health problems around the world.

Plasmodium vivax induces a moderate form of malaria, vivax malaria, characterized by periodic chills and fever, an enlarged spleen, anaemia, severe abdominal pain and headaches, and extreme lethargy. If left untreated, the disease tends to be self-limiting within a period of 10 to 30 days, but will recur periodically. Although the fatality rate of vivax malaria is low, the disease is highly debilitating and makes the patient more vulnerable to other diseases.

The incubation period ranges from 10 days to 4 weeks. Generally, paroxysms of chills and fever appear on the 14th day after the bite of an infected female anopheles mosquito. During this time the parasite has been multiplying in the liver cells of the patient. Paroxysms continue to recur every other day, as the parasite completes its 48-hour cycle of development, now in the blood. During the paroxysm, the patient first goes through a "cold stage" during which he has chilly sensations, his skin is blue, his teeth chatter and there is violent shaking. After an hour, the "hot stage" is ushered in, with a rise in temperature to as high as 107°F (41.7°C); the skin is hot and dry and the patient complains of severe headache. The fever lasts about 2 hours, and is followed by the "sweating stage", during which there is profuse perspiration, the temperature falls to normal, the headache disappears, and although weak and drowsy, the patient feels well.

Plasmodium ovale produces a disease very similar to vivax malaria.

Plasmodium malariae, the causative agent of quartan malaria, has an incubation period of 18-40 days. The paroxysms occur every 72 hours, and are longer and somewhat more severe than those accompanying vivax malaria.

Plasmodium falciparum-induced malaria (falciparum malaria) presents oedema of the brain and lungs and blockage of the kidneys, in addition to the symptoms associated with vivax malaria. Unless treated promptly, the fatality rate of falciparum malaria is high, especially in juveniles.

Paroxysms associated with falciparum malaria occur irregularly after a 12-day incubation period. They are severe, and accompanied by high temperatures. The so-called cerebral algid, haemorrhagic and pernicious types of malaria represent forms of falciparum malaria with different localizations of the parasite. In the cerebral type, the onset is delirium and coma, and death may occur in several hours without return to consciousness. "Black-water fever" or haemorrhagic malaria is a type in which haemolysis or dissolution of the red cells occurs, and dark urine due to the presence of haemoglobin is an outstanding feature. In the algid form, there are vomiting, diarrhea, and subnormal temperature.

The life cycle of the parasite and its course in the human body proceeds in the following way. The saliva of the mosquito contains the *Plasmodium* at the lance-shaped sporozoite stage of its life cycle. Upon inoculation of the host by biting, the sporozoites quickly migrate to the liver where they divide and develop into multi nucleated schizonts. Within 6 to 12 days, the schizonts disrupt and release into the blood the form known as *merozoites*. Each liver cell infected by one sporozoite releases into the blood stream from 10,000 to 30,000 merozoites. These later invade the host's erythrocytes where they grow and form more schizonts which, in turn, again divide, releasing more merozoites into the blood stream to repeat the cycle. The principal symptoms of malaria are associated with the rupture of the schizonts, the periodic lysis of the blood cells with release of merozoites and toxic wastes which cause the regular fevers and chills of malaria.

Neither vector control measures nor immuno or chemoprophylaxis have proven effective in eradicating the disease. Thus, more than ever, chemotherapy appears to be crucial in dealing with both the prevention and treatment of malaria. However, presently used drugs are constantly losing their efficacy due to the development of drug resistance by the parasite. For

example, drug resistance of *Plasmodium falciparum* to chloroquine has occurred in Bangladesh, Brazil, Burma, Colombia, Ecuador, Guyana (French), Guyana, India, Indonesia, Kampuchea, Malaysia, Nepal, Pakistan, Panama, Philippines, Surinam, Thailand, Venezuela, and Vietnam, amongst others. Therefore, the design of novel drugs is urgent.

Targets for drug design are generally nuclear-encoded gene products. However, inter-specific and developmental variation in nuclear gene expression has reduced the general efficacy of drugs which target such nuclear-encoded gene products.

Diagnosis of malaria is generally made by microscopic examination of blood films taken during episodes of fever, when the parasites may be seen. In general, the *Plasmodium* parasite is detected microscopically by examining finger prick blood samples for the presence of the morphologically distinct parasite using Giemsa stain solution (Shute *et al.*, 1980). This needs to be done by an experienced microscopist since *Plasmodium falciparum* and *Plasmodium vivax* are morphologically similar, albeit not identical. In view of the distinct epidemiologies of *P. falciparum* compared to *P. vivax*, it is important that diagnosis of infection by these species have a low error rate. Any incorrect diagnosis of falciparum malaria, for example, may be fatal for the patient. The microscopic technique is limited in so far as the method is slow and specialised personnel is required to perform the technique.

A variation of the standard microscopic assay, the quantitative buffy coat (QBC) technique is based upon the ability of parasite nucleoproteins to absorb acridine orange and fluoresce (Wardlaw *et al*, 1983). The fluorescent nucleoproteins are readily visible against a background of non-fluorescent red blood cells. Although the method is more sensitive than the standard microscopic assay, it suffers from the disadvantages associated with the standard microscopic assay. Furthermore, the requirement of costly fluorescence microscopes and centrifuges to perform the QBC assay, renders the method unrealistic in resource-limited settings which often lack even electricity.

Immunological tests, for example the ParaSight™ F test (Becton Dickinson) and the similar

ICT Malaria P.f. test (ICT Diagnostics) detect the *Plasmodium falciparum* histidine-rich protein HRP2 in blood samples derived from patients. A major drawback associated with such methods is that they require *Plasmodium falciparum* gene expression to occur before the organism can be detected. Furthermore, as considerable variation in gene expression can occur between *Plasmodium ssp.*, these tests tend to be species-specific. For example, the ParaSight™ F test (Becton Dickinson) and ICT Malaria P.f. test (ICT Diagnostics) are specific for *Plasmodium falciparum* only and incapable of detecting other species. Furthermore, these tests, in particular the ParaSight™ F test (Becton Dickinson), are subject to a high proportion of false-negative detections, such that a higher than acceptable frequency of patients infected with a *Plasmodium ssp.* go undetected.

Immunological techniques such as the enzyme-linked immunosorbent assay (ELISA) or the radio immunoassay (RIA) which detect genus- and species-specific parasite antigens also exist. However, such methods are constrained by immunological cross-reaction between parasite and host antigens on the one hand and between parasite antigens and antigens derived from other microorganisms on the other hand. As a consequence, the susceptibility of immunological methods to false positive detection of *Plasmodium* is high. As already mentioned above, species-specific detection methods lead to a large number of false-negative detections.

Furthermore, as different *Plasmodium* antigens are expressed at different developmental stages, immunological techniques may only detect the parasite at certain stages of development. Such antigenic diversity displayed by *Plasmodium* is a major obstacle to the application of immunological techniques. In addition, radioisotope-based assays such as the RIA are impractical for field use. Immunological methods cannot distinguish between past and present infections.

State-of-the art diagnostic assays, which rely on the detection of *Plasmodium* genomic DNA in a sample, are species-specific and not capable of general application for any *Plasmodium ssp.*, in part because there is considerable variation in genomic DNA between *Plasmodium*

species, such variation precluding the simultaneous detection of several *Plasmodium ssp.* in a single biological sample or alternatively, the use of a single DNA-based assay for the detection of any *Plasmodium ssp.* in a biological sample derived from a human or animal subject suspected of carrying the parasite.

As a consequence of the foregoing, there is a high demand for a reliable and simple technology for the diagnosis of *Plasmodium* in human and animal tissues.

Plasmodium ssp. possess additional genomes with potentially crucial functions (Wilson *et al.*, 1991). Until the present invention, very little was known about this extrachromosomal material. Furthermore, the function of the extrachromosomal plastid element in the protozoans remains to be determined. To date, there is no clear evidence for DNA replication or functionally active gene products from the plastid element.

SUMMARY OF THE INVENTION

One aspect of the present invention provides a method of detecting a *Plasmodium* in a biological sample derived from a human or animal subject, said method comprising contacting a *Plasmodium* extrachromosomal genetic element or a fragment thereof with said sample or nucleic acid derived therefrom for a time and under conditions sufficient for hybridisation to occur and then detecting said hybridisation using a detection means. According to this aspect, the extrachromosomal genetic element or fragment thereof may comprises a mitochondrion or mitochondrion-like molecule or a genetic sequence derived therefrom or a homologue, analogue or derivative thereof, in particular a *Plasmodium* cytochrome C oxidase (coxI) genetic sequence derived from any one of *P. falciparum, P. berghei, P. vivax, P. ovale or P. malariae*, amongst others.

Alternatively, the extrachromosomal genetic element may comprise a plastid or plastid-like molecule or a genetic sequence derived therefrom or a homologue, analogue or derivative thereof, in particular a *Plasmodium* PS1-PL470, PLH-PPH, PRB or PWQ genetic sequence derived from *P. falciparum, P. berghei, P. vivax, P. ovale* or *P. malariae*, amongst others.

Preferably, the detection means comprises a nucleic acid hybridisation reaction or polymerase chain reaction or a modification thereof, essentially as described herein.

A further aspect of the invention provides for the use of said *Plasmodium* extrachromosomal genetic element or a homologue, analogue or derivative thereof to detect *Plasmodium* in a biological sample derived from a human or animal, for example a biological sample comprising blood or blood products, in particular dried blood.

A further aspect of the invention provides an isolated extrachromosomal genetic element primer or probe derived from *Plasmodium ssp.*

A further aspect of the invention provides a kit for the detection of *Plasmodium ssp.* in a biological sample, said kit comprising one or more isolated extrachromosomal genetic element probes or primers and one or more reaction buffers suitable for use in a nucleic acid hybridisation reaction or polymerase chain reaction.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a copy of a photographic representation of an electron micrograph of the *Plasmodium berghei* extrachromosomal plastid preparation. P donates the *P.berghei* plastid while M is pBR322 (4.36 kb) used as a size marker. The bar represent the size of 1 kb.

Figure 2 is a copy of a photographic representation showing the *Eco*RI and *Hin*dIII restriction digests of *Plasmodium berghei* extrachromosomal plastid DNA. In panel (a), plastid DNA was digested with 20 units of *Eco*RI (New England Biolabs (NEB), Beverky, MA, USA) in a reaction mixture of 10 uL. The digested products were separated on a 0.4% (w/v) agarose gel at 120 V for 6 hours. Lane 1 shows Lambda Monocut markers (NEB, USA); lane 2 the *Eco*RI digest resulting in three fragments (E1, E2 and E3); lane 3 shows Lambda DNA-Hind III digest markers (NEB, USA); and lane 4 shows Lambda DNA-*Bst*EII digest markers (NEB, USA). Panel (b) shows a *Hin*dIII digest of the extrachromosomal element of *P.berghei*. The digested products were separated on a 0.6 % (w/v) agarose gel at 100 V for

6 hours. Lane 1 shows Lambda DNA-*Bst*EII digest markers (NEB, USA); lane 2 shows a *Hin*dIII digest of *P. berghei* DNA resulting in six fragments (H1, H2, H3, H4, H5 and H6); and lane 3 shows Lambda DNA-*Bst*EII digest markers (NEB, USA).

Figure 3 is a copy of a photographic representation of a Southern Hybridization of *Hin*dIII and *Hin*dIII /*Eco*RI digests of the *Plasmodium berghei* extrachromosomal element. Panel (a) shows restriction digests of *P. berghei* extrachromosomal DNA. Lane M1 shows the 123 bp DNA marker (Gibco-BRL); lane HE the *Hin*dIII /*Eco*RI digest resulting in 8 fragments (H2, H3, H4, HE1, H5, HE2, E1 and E2); lane H the *Hin*dIII digest resulting in 6 fragments, H2, H3, H4, H5 and H6); lane M2 the Lambda DNA-*Hin*dIII digest markers (NEB, USA); and lane M3 the Lambda DNA-*Bst*EII digest markers (NEB, USA). Panel (b) shows a Southern hybridization of the fragments in panel (a) with probe PS 1. Panel (c) shows a Southern hybridization of the fragments in panel (a) with probe PL470. Panel (d) shows a Southern hybridization of the fragments in panel (a) with probe PWQ.

Figure 4 is a representation of the physical and genetic map of the *Plasmodium berghei* circle. Panel (a) is a schematic representation of the arrangement of various genes and the *Eco*RI and *Hin*dIII sites are shown. The three *Eco*RI fragments, E1, E2 and E3 as well as the *Hin*dIII fragments H1, H2a, H2b as well as H4 are shown. Fragment H6 comprises of HE1, E1, E2 and HE2. The relative position of the various PCR products (Table 1) is also indicated as solid bars. Panel (b) shows a comparison between homologous genes on the *Plasmodium falciparum* and *Plasmodium berghei* plastid circles and tRNA genes are specified by a single letter amino-acid code.

Figure 5 is a copy of a photographic representation showing RT-PCR analysis of *rRNAs* transcripts. Lane 1 and 6 show the 100bp DNA ladder (Promega), lanes 2 and 3 show the RT-PCR product (L) using a set of *lsu-rRNA* gene specific primers and lanes 4 and 5 show the product (S) using a set of *ssu-rRNA* gene specific primers respectively. The (-) lanes show reactions without the reverse transcriptase enzyme.

Figure 6 is a copy of a photographic representation showing PCR amplification products generated using the primer set L1/L2 (SEQ ID NO: 5/SEQ ID NO: 6) (Panel a), and the primer set DHFR1/DHFR2 (Panel b). Blood was drawn daily for 5 days from a mouse initially infected with 5 x 10^4 parasites. Lanes 1-5 in both panels show the amplification products obtained from blood spots 1 to 5 days post-infection correspondingly. Lane 6 is the negative control with blood from an uninfected mouse and lane 7 is the positive control using 50ng of purified *P.berghei* total DNA as template. M indicates the 100bp DNA ladder (Promega) used as markers.

Figure 7 is a copy of a photographic representation showing PCR amplification of blood spots from Laotian patients diagnosed positive for *P.falciparum* malaria by Giemsa microscopy and ParaF dipstick, with the exception of one which was infected with *P. vivax* (lane 11). Primers used were the L1/L2 primer set (i.e. SEQ ID NO: 5/SEQ ID NO: 6). Lane 12 is the negative control with a blood spot from a healthy person and lane 13 is the positive control using 50ng of purified *P.falciparum* (FC27 strain) total DNA as template. M indicates the 100bp DNA ladder (Promega) used as markers. The arrow indicates the position of amplified LSU DNA.

Figure 8 is a copy of a photographic representation showing PCR amplification of blood spots from uninfected persons using the L1/L2 (SEQ ID NO: 5/SEQ ID NO: 6) primer set (Top Panel) and AC1/AC2 primer set (Lower Panel). Lanes 1 and 2 in both top and lower panels are positive controls for human β-actin using 50ng of purified total DNA from CaSki and HeLa cells. Lanes 3-10 in both top and lower panels use blood spots from uninfected persons as the template. Lanes 11 and 12 in the lower panel use blood spots from a *P.falciparum*-infected patient and a *P.vivax*-infected patient respectively. M indicates the 100bp DNA ladder (Promega) used as markers.

Figure 9 ~~is a copy of~~ a schematic representation of the aligned LSU-rRNA sequences from different *Plasmodium* species obtained from various regions in Asia. The alignment was carried out using the Clustal Method in the DNASTAR programme. Sequences indicated are

derived from several isolates of *P.falciparum* (Pf), *P.vivax* (Pv), *P.malariae* (Pm), *P. Ovale* (Po) and *P.berghei* (Pb). The alphanumeric designation following the *Plasmodium* species descriptor indicates the isolate number and geographical origin of the specimen, wherein P=Pakistan, I=India, L=Laos, C=Columbia and S=Singapore. The GenBank accession numbers for Pf(C10) and Pb(ANKA) are X95275 and U79731 respectively.

Figure 10 is a schematic representation of the aligned cox I sequences from *P.falciparum* (Pf), *P.vivax* (Pv), *P.malariae* (Pm) and *P. Ovale* (Po) isolates. The numeric designation following the *Plasmodium* species descriptor indicates the isolate number. The GenBank accession number for the *P.falciparum* sequences is M76611.

Figure 11 is a copy of a photographic representation showing PCR amplification of blood spots. Each reaction uses 1µl of blood containing different quantity of parasites. The amount of DNA used in each reaction, expressed as an equivalent number of parasites, is as follows: lane 1 contains 800 parasites; lane 2 contains 400 parasites; lane 3 contains 80 parasites; lane 4 contains 40 parasites; lane 5 contains 8 parasites; lane 6 contains 4 parasites; and lane 7 contains 0.8 parasites. Lane 8 contains the 100bp DNA ladder (Promega) used as a marker. The detection limit is 4 parasites.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In work leading up to the present invention, the inventors have discovered that the molecular composition, physical arrangements and nucleotide sequences of the extrachromosomal plastid-like element and mitochondrial element are highly conserved in different *Plasmodium ssp.*

The inventors have utilised the high degree of homology between different *Plasmodium ssp.* in the design of reliable, genera-specific or species-specific diagnostic assays for the detection of *Plasmodium*. The diagnostic assays described herein provide a significant advantage over

currently employed assays based upon the detection of *Plasmodium* genomic DNA.

Furthermore, the inventors have discovered that the assays described herein provide the added advantage of excluding the high frequency of false negative detection of *Plasmodium* in a biological sample to a greater degree than known diagnostics.

The inventors further contemplate the use of polypeptides encoded by the extrachromosomal plastid-like element, and their homologues, analogues and derivatives, as targets for drug design and in the development of anti-malarial vaccines.

Accordingly, one aspect of the invention provides a diagnostic assay for the detection of *Plasmodium* in a biological sample derived from a human or animal subject, said assay comprising the detection of a *Plasmodium* extrachromosomal genetic element or a homologue, analogue or derivative thereof in said sample.

In an alternative embodiment, the invention provides a diagnostic assay for the detection of *Plasmodium* in a biological sample derived from a human or animal subject, said assay comprising the steps of hybridising a *Plasmodium ssp.* extrachromosomal genetic element probe or primer or a homologue, analogue or derivative thereof to said sample and then detecting said hybridisation using a detection means.

According to this aspect, the *Plasmodium* detected using the invention may be any species of *Plasmodium* which carries an extrachromosomal genetic element.

In a preferred embodiment, the *Plasmodium* being detected is selected from the list comprising *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, *P. cynomolgi*, *P. gonderi*, *P. (Hepatocytis) kochi*, *P. inui*, *P. knowlesi*, *P. reichenowi*, *P. rodhaini*, *P. schwetzi*, *P. cathemerium*, *P. elongatum*, *P. relictum*, *P. lophurae*, *P. gallinaceum*, *P. yoelii*, or *P. berghei*, amongst others.

In a more particularly preferred embodiment however, the present invention is useful for the detection of a *Plasmodium* in biological samples derived from humans and the *Plasmodium* in such cases is selected from the list comprising *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*, amongst others.

The term "biological sample" as used herein shall be taken to refer to any organ, tissue, cell, exudate, nucleic acid, protein, nucleoprotein or other material which is derived from a living or once-living organism. Accordingly, biological samples may be mosquito or other vectors of *Plasmodium* ssp., human or animal tissue, blood or derivatives of blood and blood products, amongst others. A biological sample may be prepared in a suitable solution, for example an extraction buffer or suspension buffer. The present invention extends to the diagnosis of biological solutions thus prepared, the only requirement being that said solution at least comprises a biological sample as described herein.

The biological sample to be tested according to the invention, is derived from a human or animal species, in particular a human or animal which is capable of being infected by a *Plasmodium*. A particular advantage of the present invention is that it may be readily adapted to facilitate the analysis of any biological sample derived from a human or other animal. Those skilled in the relevant art will know how to modify the assay of the invention for the purposes of adapting said assay to the analysis of different biological tissues, where relevant or indicated, without any undue experimentation.

In a particularly preferred embodiment, the biological sample may be derived from the blood tissue of a human or animal subject, or cells, nucleic acid molecules and exudates derived therefrom, for example buffy coat, plasma, DNA or RNA, amongst others. The use of dried blood spots derived from human subjects as biological samples for the performance of the assays described herein is particularly contemplated by the invention.

The term "extrachromosomal genetic element" shall be taken to refer to any nucleic acid molecule, in particular DNA or RNA, which comprises a part of the complete genetic

material of a *Plasmodium ssp.* but which does not comprise a part of a *Plasmodium ssp.* chromosome or a direct gene product thereof. An extrachromosomal genetic element of a *Plasmodium ssp.* may or may not replicate independently of the *Plasmodium* genome, such that the copy number of said genetic element may vary between *Plasmodium* cells.

Accordingly, a *Plasmodium* extrachromosomal genetic element as hereinbefore defined may be a linear or circular DNA molecule. In this regard, a linear DNA molecule may resemble, at the nucleotide sequence level at least, mitochondrial DNA (Suplick *et al*, 1988), while the circular DNA molecule in a *Plasmodium* resembles a vestigial plastid genome (Gardner *et al*, 1991; Howe *et al*, 1992).

The present inventors have shown herein that the malaria parasites harbour two extrachromosomal DNAs. One of these is a small 6 kb molecule which encodes three classical mitochondrial protein coding genes, attesting to its identity. The other is a circular molecule resembling the remnant of an algal plastid genome. The mitochondria DNA of Plasmodium species is very short; about 6 kb and codes for three proteins, namely cytochrome *c* oxidase subunits I (cox I) and III (cox III), and cytochrome *b* (cyt b) as well as fragments of ribosomal RNA genes.

In a preferred embodiment of the invention, the *Plasmodium* extrachromosomal genetic element is a plastid DNA molecule comprising approximately 30-35kb of nucleotides in length.

In an alternative preferred embodiment, the *Plasmodium* extrachromosomal genetic element is mitochondrial DNA or mitochondrion-like DNA comprising approximately 6 kb in length.

In the present context, an extrachromosomal genetic element may comprise a complete organellar DNA molecule or a derivative thereof, for example a gene or an oligonucleotide which is suitable for use as a probe or primer molecule.

As used herein, the term "probe" refers to a nucleic acid molecule which is derived from a *Plasmodium* extrachromosomal genetic element and capable of being used in the detection thereof.

The term "primer" refers to a probe as hereinbefore defined which is further capable of being used to amplify a nucleotide sequence derived from a *Plasmodium* extrachromosomal genetic element in a polymerase chain reaction.

The diagnostic assay of the present invention is useful for the detection of a *Plasmodium* extrachromosomal genetic element or a *Plasmodium*-derived extrachromosomal genetic element, regardless of whether or not said genetic element expresses or is capable of expressing a polypeptide product.

The term "*Plasmodium*-derived" as used herein shall be taken to refer to an integer which, although it originates from a *Plasmodium ssp.* is not necessarily present in its natural state. For example, an extrachromosomal genetic element may be derived from a *Plasmodium ssp.* if it has been purified or partially purified and/or modified by digestion with restriction endonucleases or other DNA-modifying enzymes, to produce an analogue or derivative molecule.

The *Plasmodium* extrachromosomal genetic element probe or primer may be a mitochondrion or mitochondrion-like molecule or alternatively a plastid or plastid-like molecule, derived from a *Plasmodium ssp.* which is capable of infecting a human or animal subject.

In a particularly preferred embodiment, the extrachromosomal genetic element probe or primer or a homologue, analogue or derivative thereof, is derived from *Plasmodium berghei*, *P. ovale*, *P. malariae*, *P. falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium chabaudi*, *Plasmodium yoelii*, amongst others.

However, in a more particularly preferred embodiment of the invention, the

extrachromosomal genetic element probe or primer is at least 95% identical to one or more of the sequences set forth in SEQ ID Nos: 1-22 or Figures 9A-9J 10A-10N or a complementary nucleotide sequence, or a homologue, analogue or derivative thereof.

Alternatively, the extrachromosomal genetic element probe or primer is capable of hybridising under high stringency conditions to one or more of the sequences set forth in SEQ ID NOS: 1-22 or to any one or more of the sequences set forth in Figures 9A-9J A-10N or 10A-10N or a complementary nucleotide sequence or a homologue, analogue or derivative thereof.

In a further alternative embodiment, the *Plasmodium ssp.* extrachromosomal genetic element probe or primer used in the inventive method preferably comprises a sequence of nucleotides of at least 15 nucleotides, more preferably at least 25 nucleotides, even more preferably at least 50 nucleotides and even more preferably at least 100 nucleotides or 500 nucleotides derived from the sequence set forth in SEQ ID NOs:1-4 or to the *Plasmodium vivax, Plasmodium ovale, Plasmodium berghei, Plasmodium falciparum* or *Plasmodium malariae* sequences set forth in Figures 9 or 10, or a complement thereof.

In a most particularly preferred embodiment, the extrachromosomal genetic element probe or primer comprises a nucleotide sequence set forth in any one or more of SEQ ID NOS: 1-22 or Figures 9 or 10, or a complementary nucleotide sequence, or a homologue, analogue or derivative thereof.

For the purposes of nomenclature, the nucleotide sequences set forth in SEQ ID NOs:1-4 correspond to one strand of the PS1-PL470, PLH-PPH, PRB and PWQ genes, respectively, of the 30.7 kb *Plasmodium berghei* plastid. The inventors have shown that the extrachromosomal genetic element is transcriptionally-active, using reverse transcription polymerase chain reaction (RT-PCR), and encodes organelle-like rRNAs, tRNAs, ribosomal proteins and RNA polymerase subunits, amongst others.

The nucleotide sequences set forth in SEQ ID Nos: 5-10 and 19-20 correspond to synthetic

oligonucleotide sequences derived from the *Plasmodium berghei* plastid.

The nucleotide sequences set forth in SEQ ID Nos: 11-14 and 22 correspond to synthetic oligonucleotide sequences derived from the *Plasmodium vivax* mitochondrial coxI gene, while the nucleotide sequences set forth in SEQ ID Nos: 11, 15-18 and 21 correspond to synthetic oligonucleotide sequences derived from the *P. falciparum* mitochondrial coxI gene.

The nucleotide sequences Pm1/S and Pm38/S in Figure 9A-9J relate to the plastid-like extrachromosomal genetic element in two *P.malariae* isolates and Po35/S and Po36/S relate to the extrachromosomal genetic element of two *P.ovale* isolates. The nucleotide sequences designated Pv12/P, Pv13/P, Pv15/I, Pv16/L, Pv17/S and Pv86/C in Figure 9 relate to plastid-like extrachromosomal genetic element sequences of different *P.vivax* isolates.

For the present purpose, "homologues" of a nucleotide sequence shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as the nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence within said sequence, of one or more nucleotide substitutions, insertions, deletions, or rearrangements.

"Analogues" of a nucleotide sequence set forth herein shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as a nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence of any non-nucleotide constituents not normally present in said isolated nucleic acid molecule, for example carbohydrates, radiochemicals including radio nucleotides, reporter molecules such as, but not limited to biotin, DIG, alkaline phosphatase or horseradish peroxidase, amongst others.

"Derivatives" of a nucleotide sequence set forth herein shall be taken to refer to any isolated nucleic acid molecule which contains significant sequence similarity to said sequence or a part thereof. Generally, the nucleotide sequence of the present invention may be subjected to

mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or insertions. Nucleotide insertional derivatives of the nucleotide sequence of the present invention include 5′ and 3′ terminal fusions as well as intra-sequence insertions of single or multiple nucleotides or nucleotide analogues. Insertional nucleotide sequence variants are those in which one or more nucleotides or nucleotide analogues are introduced into a predetermined site in the nucleotide sequence of said sequence, although random insertion is also possible with suitable screening of the resulting product being performed. Deletional variants are characterised by the removal of one or more nucleotides from the nucleotide sequence. Substitutional nucleotide variants are those in which at least one nucleotide in the sequence has been removed and a different nucleotide or nucleotide analogue inserted in its place.

The present invention encompasses all such homologues, analogues or derivatives of a *Plasmodium* extrachromosomal genetic element, subject to the proviso that said homologues, analogues or derivatives are useful in the performance of at least one assay format as described herein.

According to this aspect of the invention, the *Plasmodium* extrachromosomal genetic element probe or primer may comprise inosine, adenine, guanine, thymidine, cytidine or uracil residues or functional analogues or derivatives thereof which are capable of being incorporated into a polynucleotide molecule, provided that the resulting probe or primer is capable of hybridising under at least low stringency conditions to a *Plasmodium* extrachromosomal genetic element.

The inventors have discovered that the extrachromosomal genetic element of *Plasmodium* is particularly useful as a marker of *Plasmodium* infection in a human or animal subject, because the detection of said element is not subject to the disadvantages of other assay methods, in particular the prevalence of false negative detection. As a consequence, fewer numbers of *Plasmodium*-infected hosts escape detection, by screening such hosts for the presence of the extrachromosomal genetic element according to the embodiments described herein (1% or less false negative detection compared to 3% or more for other methods), than

by screening for the presence of other *Plasmodium*-expressed genes or by screening for the expression products of said genes.

Furthermore, the present invention is a procedure for assaying or identifying *Plasmodium* in a biological sample, preferably blood or a derivative of blood and in particular a biological sample which comprises dried blood.

The present invention clearly contemplates diagnostic assays which are capable of both genera-specific or species-specific detection. Accordingly, in one embodiment, the *Plasmodium ssp.* extrachromosomal genetic element probe or primer or a homologue, analogue or derivative thereof comprises DNA capable of being used to detect multiple *Plasmodium ssp.* In an alternative embodiment, the *Plasmodium ssp.* extrachromosomal genetic element probe or primer or a homologue, analogue or derivative thereof comprises DNA capable of being used to detect a particular *Plasmodium ssp.*

The inventors have discovered further that the coding region of a *Plasmodium* extrachromosomal genetic element is highly-conserved in different *Plasmodium ssp.*, while there is much more variation at the nucleotide level in the non-coding regions. Whilst not being bound be any theory or mode of action, the more highly conserved sequences in the extrachromosomal genetic element derived from a particular species of *Plasmodium* are particularly useful as genera-specific probes and/or primers for the detection of any *Plasmodium*, while the less-conserved sequences of said element may be useful as species-specific probes and/or primers for the detection of a sub-group of *Plasmodium*, for example a sub-group which infects humans or primates as opposed to other animals, or which induces a specific form of malaria in humans.

The present inventors have also shown herein that certain sequences of the *Plasmodium* cytochrome *c* oxidase differ between species. Accordingly, a preferred embodiment of the present invention extends to the use of nucleotide sequences derived from the mitochondrial extrachromosomal genetic element of *Plasmodium*, more preferably derived from *P.*

falciparum or *P. vivax* in the diagnosis of species-specific infections by one or more of *P. malariae*, *P. ovale*, *P. vivax* or *P. falciparum*, amongst others.

According to this embodiment, the nucleotide sequence set forth in SEQ ID NO:11 is a "universal probe" for the detection of at least *P. falciparum* and *P. vivax*, whilst SEQ ID Nos: 21 and 22 are species-specific probes for the detection of *P. falciparum* and *P. vivax*, respectively. Particularly preferred primer combinations for the species-specific detection of *P. falciparum* include, but are not limited to primers comprising SEQ ID Nos:7 and 8, SEQ ID Nos: 11 and 15, SEQ ID Nos: 11 and 16, SEQ ID Nos:11 and 17 SEQ ID Nos:16 and 18 and alternative combinations thereof readily determined by those skilled in the art. Particularly preferred primer combinations for the species-specific detection of *P. vivax* include, but are not limited to primers comprising SEQ ID Nos:11 and 12 and SEQ ID Nos:13 and 14 and alternative combinations thereof readily determined by those skilled in the art.

Furthermore, one or more of the diagnostic assays described herein may also be adapted to a genera-specific or species-specific assay by varying the stringency of the hybridisation step. Accordingly, a low or lower stringency hybridisation may be used to detect several different species of *Plasmodium* in one or more biological samples being assayed, while a high or higher stringency of hybridisation is used to detect the presence of a specific species of *Plasmodium*.

For the purposes of defining the level of stringency, a low stringency is defined herein as being a hybridisation and/or a wash carried out in 6xSSC buffer, 0.1% (w/v) SDS at 28°C. A moderate stringency is defined herein as being a hybridisation and/or wash carried out in 2xSSC buffer, 0.1% (w/v) SDS at a temperature in the range 45°C to 65°C. A high stringency is defined herein as being a hybridisation and/or wash carried out in 0.1xSSC buffer, 0.1% (w/v) SDS at a temperature of at least 65°C. Those skilled in the art will be aware of equivalent reaction conditions to those described herein for defining the hybridisation stringency.

Generally, the stringency is increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS and/or increasing the temperature of the hybridisation and/or wash. Those skilled in the art will be aware that the conditions for hybridisation and/or wash may vary depending upon the nature of the hybridisation membrane or the type of hybridisation probe used. Conditions for hybridisations and washes are well understood by one normally skilled in the art. For the purposes of clarification of the parameters affecting hybridisation between nucleic acid molecules, reference is found in pages 2.10.8 to 2.10.16. of Ausubel *et al.* (1987), which is herein incorporated by reference.

The detection means according to this aspect of the invention may be any nucleic acid-based detection means, for example nucleic acid hybridisation techniques or paper chromatography hybridisation assay (PACHA) or an amplification reaction such as a polymerase chain reaction (PCR) or nucleic acid sequence-based amplification (NASBA) system. The invention further encompasses the use of different assay formats of said nucleic acid-based detection means, including restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), single-strand chain polymorphism (SSCP), amplification and mismatch detection (AMD), interspersed repetitive sequence polymerase chain reaction (IRS-PCR), inverse polymerase chain reaction (iPCR) and reverse transcription polymerase chain reaction (RT-PCR), amongst others.

Wherein the detection means is a nucleic acid hybridisation technique, the *Plasmodium* extrachromosomal genetic element probe may be labelled with a reporter molecule capable of producing an identifiable signal (e.g. a radioisotope such as ^{32}P or ^{35}S or a biotinylated molecule). According to this embodiment, those skilled in the art will be aware that the detection of said reporter molecule provides for identification of the *Plasmodium* extrachromosomal genetic element probe and that, following the hybridisation reaction, the detection of the corresponding *Plasmodium ssp.* extrachromosomal genetic element in the biological sample is facilitated. Those skilled in the art will recognise that additional probes may be used to confirm the assay results obtained using a single probe.

A variation of the nucleic acid hybridisation technique contemplated by the present invention is the paper chromatography hybridisation assay (PACHA) described by Reinhartz *et al.* (1993) and equivalents thereof, wherein a target nucleic acid is labelled with a reporter molecule such as biotin, applied to one end of a nitrocellulose or nylon membrane filter strip and subjected to chromatography under the action of capillary or other forces (eg. an electric field) for a time and under conditions sufficient to promote migration of said target nucleic acid along the length of said membrane to a zone at which a *Plasmodium* extrachromosomal genetic element DNA probe is immobilised thereto, for example in the middle region. According to this detection format, labelled target nucleic acid comprising a *Plasmodium* extrachromosomal genetic element which is complementary to the probe will hybridise thereto and become immobilised in that region of the membrane to which the probe is bound. Non-complementary sequences to the probe will diffuse past the site at which the probe is bound. Those skilled in the art will be aware that the target nucleic acid may comprise a crude or partially-pure extract of *Plasmodium* DNA or RNA or alternatively, comprise amplified DNA or purified *Plasmodium* extrachromosomal genetic element DNA. Additional variations of this detection means which utilise the nucleotide sequences described herein are clearly encompassed by the present invention.

Wherein the detection means is an RFLP, nucleic acid derived from the biological sample, in particular DNA, is digested with one or more restriction endonuclease enzymes and the digested DNA is subjected to electrophoresis, transferred to a solid support such as, for example, a nylon or nitrocellulose membrane, and hybridised to the *Plasmodium* extrachromosomal genetic element probe as hereinbefore defined, optionally labelled with a reporter molecule. According to this embodiment, a specific pattern of DNA fragments is hybridised to the *Plasmodium* extrachromosomal genetic element probe, said pattern optionally specific for a particular *Plasmodium ssp.*, to enable the user to distinguish between different species of the parasite.

Wherein the detection means is an amplification reaction for example a polymerase chain reaction or a nucleic acid sequence-based amplification (NASBA) system or a variant of same,

one or more nucleic acid primer molecules of at least 15 contiguous nucleotides in length derivable from the *Plasmodium* extrachromosomal genetic element as hereinbefore defined, or its complementary nucleotide sequence or a homologue, analogue or derivative thereof, is hybridised to the biological sample comprising nucleic acid or alternatively, to nucleic acid derived from said sample and nucleic acid copies of the *Plasmodium* extrachromosomal genetic element present in said sample or a part or fragment thereof are enzymically-amplified.

Those skilled in the art will be aware that there must be a sufficiently high percentage nucleotide sequence identity between the *Plasmodium* extrachromosomal genetic element primers and the sequences in the template molecule to which they hybridise. As stated previously, the hybridisation conditions may be varied to promote hybridisation.

Preferably, the *Plasmodium* extrachromosomal genetic element primer is at least 95% identical to the complement of the nucleotide sequence in the template molecule to which it hybridises. More preferably, each *Plasmodium* extrachromosomal genetic element primer is substantially the same as the complement of the nucleotide sequence in the template molecule to which it hybridises.

Preferably, the *Plasmodium* extrachromosomal genetic element primer is contained in an aqueous mixture of other nucleic acid primer molecules. More preferably, the nucleic acid primer molecule is in a substantially pure form.

The *Plasmodium* extrachromosomal genetic element primer preferably comprises the sequence of nucleotides set forth in any one or more of SEQ ID Nos: 5-22 or Figures 9A-9J or 10A-10N or a complementary strand or a homologue, analogue or derivative thereof.

In a more particularly preferred embodiment, the *Plasmodium* extrachromosomal genetic element primers are hybridised to a *Plasmodium* extrachromosomal genetic element contained in the biological sample being analysed, as probe pairs, in the combinations comprising SEQ

ID Nos: 5 and 6; or SEQ ID Nos: 7 and 8; or SEQ ID Nos: 9 and10; or SEQ ID Nos. 11 and 12; or SEQ ID Nos: 11 and 15; or SEQ ID Nos: 11 and 16; or SEQ ID Nos: 11 and 17; or SEQ ID Nos: 13 and 14; or SEQ ID Nos: 16 and 18; or SEQ ID Nos:21 and 22 or complementary strands, homologues, analogues or derivatives thereof.

The present invention particularly contemplates the use of primers as set forth in any one or more of SEQ ID Nos:11-18 as being useful in the differentiation of *Plasmodium* species as well as for detecting *Plasmodium* in a biological sample.

The *Plasmodium* extrachromosomal genetic element present in the biological sample, or a part or fragment thereof which is enzymically-amplified, is defined herein as a "template molecule". The template molecule may be a genetic sequence which is at least 40% identical at the nucleotide sequence level to SEQ ID Nos: 1-4 or to its complementary nucleotide sequence or to the *P. vivax, P. ovale, P. berghei, P. falciparum* or *P. malariae* sequences set forth in Figure 9A-9J or Figure 10, the only requirement being that it comprises a *Plasmodium* extrachromosomal genetic element primer as hereinbefore defined.

Those skilled in the art will also be aware that, in one format, the polymerase chain reaction provides for the hybridisation of non-complementary *Plasmodium* extrachromosomal genetic element primers to different strands of the template molecule, such that the hybridised primers are positioned to facilitate the 5'- 3' synthesis of nucleic acid in the intervening region, under the control of a thermostable DNA polymerase enzyme. As a consequence, the polymerase chain reaction provides an advantage over other detection means in so far as the nucleotide sequence in the region between the hybridised *Plasmodium* extrachromosomal genetic element primers may be unknown and unrelated to any known nucleotide sequence.

In a particularly preferred embodiment, the nucleic acid template molecule comprises, in addition to other nucleotide sequences, a sequence of nucleotides derived from or contained

within any one or more of the sequences set forth in SEQ ID Nos: 1-18 or a complementary sequence or a homologue, analogue or derivative thereof.

In an alternative embodiment, wherein the detection means is AFLP, the *Plasmodium* extrachromosomal genetic element primers are selected such that, when nucleic acid derived from the biological sample, in particular DNA, is amplified, different length amplification products are produced from different *Plasmodium ssp.* The amplification products may be subjected to electrophoresis, transferred to a solid support such as, for example, a nylon or nitrocellulose membrane, and hybridised to the *Plasmodium* extrachromosomal genetic element probe as hereinbefore defined, optionally labelled with a reporter molecule. According to this embodiment, a specific pattern of amplified DNA fragments is hybridised to the *Plasmodium* extrachromosomal genetic element probe, said pattern optionally specific for a particular *Plasmodium ssp.*, to enable the user to distinguish between different species of the parasite in much the same way as for RFLP analysis.

The technique of AMD facilitates, not only the detection of a *Plasmodium* extrachromosomal genetic element in a biological sample, but also the determination of nucleotide sequence variants which differ from the *Plasmodium* extrachromosomal genetic element primers and probes used in the assay format.

Wherein the detection means is AMD, the *Plasmodium* extrachromosomal genetic element probe is end-labelled with a suitable reporter molecule and mixed with an excess of the amplified template molecule. The mixtures are subsequently denatured and allowed to renature to form nucleic acid "probe:template hybrid molecules" or "hybrids", such that any nucleotide sequence variation between the probe and the temple molecule to which it is hybridised will disrupt base-pairing in the hybrids. These regions of mismatch are sensitive to specific chemical modification using hydroxylamine (mismatched cytosine residues) or osmium tetroxide (mismatched thymidine residues), allowing subsequent cleavage of the modified site using piperidine. The cleaved nucleic acid may be analysed using denaturing polyacrylamide gel electrophoresis followed by standard nucleic acid hybridisation as

described *supra* to detect the *Plasmodium* extrachromosomal genetic element nucleotide sequences.

Those skilled in the art will be aware of the means of end-labelling a genetic probe according to the performance of the invention described in this embodiment.

According to this embodiment, the use of a single end-labelled probe allows unequivocal localisation of the sequence variation. The distance between the point(s) of sequence variation and the end-label is represented by the size of the cleavage product.

In an alternative embodiment of AMD, the probe is labelled at both ends with a reporter molecule, to facilitate the simultaneous analysis of both DNA strands.

Wherein the detection means is IRS-PCR, the *Plasmodium* extrachromosomal genetic element primers are selected such that they each include one highly-repetitive restriction enzyme cleavage site, for example *Alu*I, which is ubiquitous in many genomes. According to this embodiment, the appropriate restriction enzyme cleavage site is selected such that it is ubiquitous in *Plasmodium* extrachromosomal genetic element nucleotide sequences. The amplified template DNA is electrophoresed under conditions which facilitate high resolution and optionally probed with a labelled *Plasmodium* extrachromosomal genetic element probe.

Optionally, the amplified template DNA may be end-filled using Klenow fragment of DNA polymerase I or other suitable means, prior to the electrophoresis step.

According to this embodiment, different combinations of primers produce different patterns of amplified template nucleic acid.

Furthermore, with any primer combination used, each *Plasmodium ssp.* will produce a distinctive pattern of amplified template nucleic acid. As a consequence, the detection means is suitable for distinguishing between different *Plasmodium ssp.*, in addition to being useful

for the detection of the *Plasmodium* extrachromosomal genetic element *per se* in a biological sample.

Wherein the detection means is RT-PCR, the nucleic acid sample comprises an RNA molecule which is a transcription product of the *Plasmodium* extrachromosomal genetic element DNA or a homologue, analogue or derivative thereof. As a consequence, this assay format is particularly useful when it is desirable to determine expression of one or more *Plasmodium* extrachromosomal genetic element genes.

According to this embodiment, the RNA sample is reverse-transcribed to produce the complementary single-stranded DNA which is subsequently amplified using standard procedures.

Variations of the embodiments described herein are described in detail by McPherson *et al.* (1991), which is incorporated in the references.

The present invention clearly extends to the use of any and all detection means referred to *supra* for the purposes of diagnosing *Plasmodium* infection in humans and other animals.

The amplification reaction detection means described *supra* may be further coupled to a classical hybridisation reaction detection means to further enhance sensitivity and specificity of the inventive method, in particular by hybridising the amplified DNA with a *Plasmodium* extrachromosomal genetic element probe which is different from any of the *Plasmodium* extrachromosomal genetic element primers used in the amplification reaction.

Accordingly, a particularly preferred embodiment of the inventive method comprises the further step of detecting the amplified nucleic acid by contacting one or more of the nucleotide sequences set forth in SEQ ID Nos:19-22 thereto for a time and under conditions sufficient for hybridisation to occur.

Similarly, the hybridisation reaction detection means described *supra* may be further coupled to a second hybridisation step employing a *Plasmodium* extrachromosomal genetic element probe which is different from the probe used in the first hybridisation reaction.

The nucleotide sequences set forth in SEQ ID Nos: 19-22 are particularly suited to the performance of this embodiment, however those skilled in the art would readily be able to utilise the nucleotide sequences provided by the present invention in the performance of this embodiment. In particular, SEQ ID Nos:19 and 20 enable the identification of LSU and SSU sequences, respectively in a *Plasmodium ssp.*, whilst SEQ ID Nos: 21 and 22 may be used for the specific detection of amplified or hybridised coxI genetic sequences derived from *Plasmodium falciparum* and *P. vivax*, respectively.

A further aspect of the invention provides an isolated extrachromosomal genetic element probe or primer derived from *Plasmodium ssp*, or a homologue, analogue or derivative thereof, according to the embodiments described herein.

Preferably, the extrachromosomal genetic element probe or primer is derived from a *Plasmodium ssp.* selected from the list comprising *P. berghei, P. falciparum, P. vivax, P. malariae, P. ovale, P. cynomolgi, P. gonderi, P. (Hepatocytis) kochi, P. inui, P. knowlesi, P. reichenowi, P. rodhaini, P. schwerzi, P. cathemerium, P. elongatum, P. relictum, P. lophurae, P. gallinaceum* or *P. yoelii*, amongst others.

In a particularly preferred embodiment, the extrachromosomal genetic element probe or primer is derived from *P. falciparum, P. berghei, P. ovale, P.vivax* or *P.malariae*. More particularly, the extrachromosomal genetic element probe or primer comprises a sequence of nucleotides which is at least 95% identical to the sequence set forth in any one or more of SEQ ID Nos: 1-22, or any one or more of the *P. berghei, P. ovale, P.vivax* or *P.malariae* sequences set forth in Figure 9 or the *P. falciparum, P. ovale, P.vivax* or *P.malariae* sequences set forth in Figure 10 or any one or more of the sequences set forth or a complementary nucleotide sequence, homologue, analogue or derivative thereof which is

at least useful as a primer or probe for the diagnosis of infection of a human or animal subject by a *Plasmodium ssp.*

Alternatively, the probe or primer at least comprises a nucleotide sequence which is capable of encoding an amino acid sequence which is encoded by one or more of SEQ ID Nos:1-4 or a nucleotide sequence set forth in Figure 9A-9J or 10A-10N or a complementary sequence thereto.

Wherein the extrachromosomal genetic element is a plastid or plastid-like molecule, it is preferred that it be derived from a species of *Plasmodium* other than *P. falciparum*.

A further aspect of the present invention contemplates a kit for convenient detection of a *Plasmodium ssp.* in a biological sample.

In an alternative embodiment, the kit of the present invention is also useful for convenient assay of infection by a *Plasmodium ssp.* parasite, wherein the sample being tested is derived from a human or other animal or mosquito suspected of being infected with said parasite.

The kit of the present invention is compartmentalized to contain in a first compartment, one or more nucleic acid molecules which comprise a sequence of nucleotides corresponding to a *Plasmodium* extrachromosomal genetic element or a complementary nucleotide sequence or a homologue, analogue or derivative thereof as hereinbefore defined.

In a preferred embodiment, the first compartment is adapted to contain one or more nucleic acid molecules which are at least 95% identical to the nucleotide sequence set forth in any one or more of SEQ ID Nos: 1-22 or any one or more of the *Plasmodium vivax, P. ovale, P. falciparum, P. berghei* or *P. malariae* sequences set forth in Figure 9A-9J and/or Figure 10A-10N or its complement or a derivative, homologue or analogue thereof. In a more preferred embodiment, the kit at least comprises one or more of the probe or primer sequences as set forth in any one of SEQ ID Nos: 5-22. The selection of SEQ ID Nos:21 and/or 22 as a probe is particularly suited to species-specific detection assay formats.

In a particularly preferred embodiment, the subject kit comprises a first primer and a second primer for the amplification of nucleic acid derived from or related to a *Plasmodium* extrachromosomal genetic element, such as a mitochondrion or plastid-like element. According to this embodiment, the first primer preferably comprises a sequence selected from SEQ ID Nos: 5, 7, 9, 11, 14 or 18 and the second primer preferably comprises a sequence selected from SEQ ID Nos: 6, 8, 10, 12, 13 or 15-17 or a derivative thereof.

In a more particularly preferred embodiment, the first and second primers comprise the sequences set forth in SEQ ID Nos: 5 and 6, or SEQ ID Nos: 11 and 12 or SEQ ID Nos: 11 and 15 or SEQ ID Nos: 11 and 16 or SEQ ID Nos: 11 and 17 or SEQ ID Nos: 13 and 14 or SEQ ID Nos: 16 and 18, respectively. These combinations are particularly suited to species-specific detection assay formats.

The invention clearly extends to kits at least comprising one or more pairs of said primers.

The invention extends further to such kits wherein both primers of a primer pair are provided in the same compartment, in aqueous solution or dried, such that the subject primers are at a relative concentration suitable for subsequent use in an amplification reaction.

The kit optionally comprises several second containers comprising a reaction buffer suitable for use in one or more of the detection means described herein and optionally several third containers comprising a nucleic acid molecule positive standard, to which the assay sample result may be compared.

In an exemplified use of the subject kit, a negative control reaction is carried out in which the contents of the first container are contacted with the contents of the second container. At the same time, the sample to be tested is contacted with the contents of the first and second containers for a time and under conditions sufficient for hybridisation to occur. If the reagents contained in the first container provided are not labelled with a reporter molecule, then the contents of the first container may be so labelled prior to the hybridisation reaction being carried

out. The hybridised test sample and the negative control sample are then subjected to a detecting means as hereinbefore described. In analysing the results obtained using said kit, the control negative control reaction, test sample and nucleic acid molecule positive standard are compared side-by-side. The contents of the third container should always provide a positive result upon which to compare the results obtained for the negative control and test sample. If the results of the test sample are identical to the results obtained for the negative control, then the biological sample does not contain a *Plasmodium ssp.* extrachromosomal genetic element. However, if the test sample produces a nucleic acid molecule which is similar or the same as that contained in the positive standard, albeit of different intensity, then the biological sample contains a *Plasmodium ssp.* extrachromosomal genetic element.

The kit may further comprise additional probes and/or primers for the purpose of detecting amplified or hybridised nucleic acid in additional rounds of hybridisation and/or amplification.

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

Preparation of *Plasmodium berghei* extrachromosomal DNA

Plasmodium berghei (ANKA strain) was maintained in Swiss White mice by continuous blood passage. Development of parasitemia was monitored daily by thin blood film analysis (Shute, 1988). Parasites were obtained by lysis of infected red blood cells with 1% saponin. The extrachromosomal element was purified from the parasites using a modified procedure of the Qiagen plasmid mini preparation kit (Qiagen Inc., Chatsworths, CA, USA). Parasites from 10 infected mice (20-25 g) with a parasitemia of 60% were resuspended in 5 ml of P1 buffer, lysed with 5 ml of P2 buffer and neutralised with 5 ml of P3 buffer. After chilling on ice for 20 minutes, the precipitate was removed by centrifugation according to the manufacturer's recommendation and 200 μl of proteinase K (50 mg/ml) were added to the supernatant, which was then reincubated for 2 hours at 37 C. The supernatant was

subsequently passed through a tip-20 Qiagen column which had previously been equilibrated with 1 ml of QBT buffer. The column was washed four times with 1 ml of QC buffer each. Finally, the extrachromosomal element was eluted with 1 ml of QF - buffer which was preheated to 65 C. The DNA was precipitated with isopropanol, washed with 70% ethanol, dried and dissolved in 25 μL of TE buffer.

EXAMPLE 2

Preparation and restriction digest of *P. berghei* extrachromosomal DNA

Plasmodium berghei extrachromosomal DNA was extracted from the parasite using the Qiagen plasmid mini preparation kit (Qiagen Inc., Chatsworths, CA, USA). Electron microscopic analysis of this preparation showed circular DNA elements of about 10 times the size of control pBR 322 plasmids (Figure 1). The preparation was not homogenous and, in addition to the circular elements many linear molecules of different lengths were observed. The preparation is enriched for the extrachromosomal DNA elements of both circular and linear DNA representing the homologues of the 35 kb circle and 6 kb mitochondrial DNA. These are likely to be 6 kb DNA molecules which are tandemly arrayed in head-to-tail configurations.

The extracted plastid DNA was digested into 3 fragments of 15 kb (E3), 10 kb (E2) and 5.7 kb (E1) by *EcoR*I (Figure 2a). This gives the plastid an estimated size of 31 kb. *Hind* III digest of the DNA yielded 6 fragments of 22.4 kb (H6), 4.4 kb (H5), 1.85 kb (H4), 1.23 kb (H3), 0.95 kb (H2) and 0.7 kb (H1), respectively (Figure 2b). H3 and H5 are fragments from the mitochondrial 6 kb genome.

The estimated size of the *P. berghei* circle is 31 kb according to Electron microscopic measurements using pBR322 as control as well as from size calculations using the *EcoR* I restriction digest fragments.

Extrachromosomal circular DNA has not only been found in *Plasmodium* species but also in

other parasitic protozoa such as *Babesia* and *Entamoeba* (Gozar and Bagnara, 1995; Egea and Lang-Unnasch, 1995; Sehgal et al., 1994) suggesting a common evolutionary origin of this circular DNA material (Williamson et al. 1994). By maintaining such extrachromosomal information during evolution it appears that this highly conserved and seemingly functional extrachromosomal DNA molecule is important for parasite development and that knowledge of its functions will greatly aid in providing novel targets for drug development.

Our preliminary tests using an antisense oligonucleotide approach indicate that this extrachromosomal element may indeed be crucial for parasite survival.

EXAMPLE 3

PCR amplification and sequence analysis of plastid DNA.

In order to obtain a genetic map of the approximately 35 kb *Plasmodium berghei* extrachromosomal plastid, polymerase chain reaction (PCR) amplifications and sequence analysis of plastid DNA were carried out.

PCR was performed using the United States Biochemical (Amersham) PCR kit in a 100 μl reaction mixture containing 2 mM $MgCl_2$, 0.2 mM of each dNTP, 4 ng/μL of each primer, 5 units of *Taq* DNA polymerase, 10 μL of the 10 X PCR buffer and 1 μL of the extrachromosomal DNA prepared as described in Examples 1 and 2. A "hot start" was carried out at 95°C for 5 minutes without the dNTPs and Taq polymerase. This was followed by the addition of dNTPs and Taq polymerase and 40 cycles of denaturation (90°C, 1.5 minutes), annealing (55°C, 3 minutes) and extension (72°C, 5 minutes). A final extension was performed at 72°C for 10 minutes.

The PCR products were loaded onto a 1 % low melting point agarose gel, extracted by the freeze-thaw method (Shoemaker and Salyers, 1990) and then cloned into the Promega pGEM-T vector.

Clone H2a was constructed by cloning the second fragment of a *Hin*dIII digest of the

extrachromosomal element into the pBluescript vector. (Stratagene, USA).

The clones were sequenced using the ABI PRISM Dye terminator cycle sequencing kit from Perkin-Elmer on the 373A DNA sequencer from Applied Biosystems. The percentage homology with the P. falciparum extrachromosomal element (Accession No. X 95275 and X 95276) was obtained using the Martinez/Needleman-Wunsch DNA alignment programme from DNASTAR.

PCR amplification of different parts of the extrachromosomal plastid were performed using primer sets homologous to sequences from the 35 kb circle of *P.falciparum* (Table 1). These include the primer sets comprising SEQ ID Nos: 5 and 6 (L/L Primer set), SEQ ID Nos: 7 and 8 (L/S primer pair) and SEQ ID Nos: 9 and 10 (S/S Primer pair, homologous to the small-subunit (ssu) -*rRNA* of *P. falciparum*).

The amplified regions obtained with these primers lay within the large subunit (lsu) -*rRNA* gene, *rpo B* gene, the cluster of 10 *tRNAs*, part of the cluster of four *tRNAs* located close to the 3' end of the *tufA* gene in *P. falciparum* as well as the region between the *lsu-rRNA* and the *ssu-rRNA* genes.

All PCR fragments were cloned into the pGem-T vector from Promega. Sequence analysis performed using the Martinez/Needleman-Wunsch DNA alignment on all clones which had been purified using Qiagen midi plasmid preparation columns showed a similarity index of greater than 80% with the *P.falciparum* circle except for the PPH and PWQ fragment (Table 1). The PRB fragment was homologous to the *P.falciparum rpo B* gene with a similarity index of 87.9% for the DNA sequence and 85.6% for the corresponding amino acid sequence (using the Lipman-Pearson protein alignment). The PPH sequence spanning the cluster of 10 *tRNA* genes had a similarity index of only 78%. While the *tRNA* coding regions were highly similar to those in *P.falciparum* the non-coding spaces were much less conserved between the two Plasmodium species.

In order to examine if the *lsu-rRNA* gene in *P. berghei* exists as a repeat, a single forward primer (L3) homologous to the 3' end of the *P.falciparum lsu-rRNA* sequence and 2 distinct reverse primers homologous to the ORF 470 (04) and the start of the cluster of 10 *tRNA* genes (3H) of the *P.falciparum* circle were designed. The fragment amplified with the L3/04 primer set (PL470) was distinct from that amplified using the L3/3H set (PLH). Sequence analysis of PL470 showed a homology of 83% with the same region in *P. falciparum*. The sequence of PLH was homologous to the 3' end of the *lsu-rRNA* and the 3' end of *rps 4* of the *P.falciparum* circle (data not shown) indicating that the *lsu-rRNA* gene exists as a repeat in *P.berghei*. In addition, a cluster of intervening *tRNA* genes was present between the *lsu-rRNA* and *ssu-rRNA* genes (fragment PLS). This repetition and arrangement is similar to the organisation of the *P.falciparum* circle, where a palindromic sequence of genes for the subunit *rRNAs* and several *tRNAs* exists. Each arm of the palindrome contains one *ssu* and one *lsu-rRNA* gene and a cluster of intervening *tRNA* genes (Gardner *et al.*, 1993).

From the nucleotide sequences set forth in SEQ ID Nos: 1-4, it is clear that the genes in the *P.berghei* circle are homologous to those in the 35 kb *P.falciparum* circle. Major differences in sequence are observed in the non-coding spaces between tRNA gene clusters. The arrangement of genes appears to be similar in both Plasmodium species and a repeat of the rRNA genes does not only exist in *P.falciparum* but also in the *P.berghei* circle.

EXAMPLE 4

Southern Hybridisation of restriction fragments

Fragments from the *Hin*dIII and *Hin*dIII/ *Eco*RI digests of the *Plasmodium berghei* extrachromosomal element were separated on a 1 % (w/v) agarose gel at 120 V for 4 hours. The separated fragments were then transferred onto a Nylon membrane (Hybond-N, Amersham) by capillary action using 20x SSC buffer (0.3M sodium citrate, 3M sodium chloride, pH 7.0). Southern hybridization was performed using probes specific for the approximately 35 kb circle that were made from the cloned PCR products, according to the preceding Examples.

The PCR products were liberated from the vector by digestion with *Apa*I and *Pst*I. The enhanced chemiluminescence (ECL) direct nucleic acid labelling and detection system (Amersham International PLC, England) was used for labelling the probe, for hybridisation and for detection.

Each of these steps were carried out according to the manufacturer's instruction. First, 8 μg of probe in a volume of 20 μL were denatured by boiling for 5 minutes and immediately cooled on ice for 5 minutes. 20 μL of labelling reagent were then added. This was followed by the addition of 20 μL of glutaraldehyde solution. The mixture was incubated for 20 minutes at 37°C before addition to the hybridization buffer. The ECL Gold hybridisation buffer containing 0.5M NaCl and 5% blocking agent was used for hybridisation. The blots were prehybridised for 2 hours at 42°C and the labelled probe was added to a final concentration of 800 ng DNA/ml. Hybridisation was allowed to proceed overnight at 42°C. The blots were washed twice in primary wash buffer containing 6M urea, 0.4% SDS and 0.5x SSC at 42°C for 20 minutes. This was followed by two rounds of washing in 2x SSC buffer at room temperature for 10 minutes. For detection, 6.5 ml of equal volumes of detection reagents 1 and 2 were mixed and added to the blot for 1 minute. The blot was then drained, wrapped in Saran Wrap and the DNA side was exposed to an autoradiography film.

A double digest of the *P. berghei* circle with *Hin*dIII followed by *Eco*RI resulted in the following fragments: 10kb (E2), 5.7kb (E1), 5.0kb (HE2), 4.4kb (H5), 2.3kb (HE1), 1.85 (H4), 1.23 (H3), 0.95(H2) and 0.7kb (H1). The PS 1 probe hybridised to H2, the PL470. probe hybridised to H6 and HE2 while the PWQ probe hybridised to H6 and E2 (Fig. 3).

The results obtained with various other probes are shown in Table 2. Of interest to note is that H2 contained 2 distinct fragments which hybridised with PS 1 and PL3. One of the H2 fragments (H2a) was cloned into Bluescript vector (pBS KS (II)+) and sequenced. The sequence corresponded to the internal region of the *P.falciparum* 35 kb ssu-rRNA gene (Table 1, sequence H2a). The other fragment (H2b) arose from the two Hind III sites within the lsu-rRNA gene. The presence of these two sites was confirmed by the sequences from the

Table 1. Description of clones of various segments from the extrachromosomal element in *P. berghei* and their percentage homology with *P. falciparum*.

Name of clone	Description	Size (bp)	Percentage homology with *P. falciparum*	*Eco*RI/*Hind*III sites
H2a	Second fragment of *Hind*III digest containing SSU rRNA	949	92.3	Two *Hind*III sites
PS1	PCR product of SSU rRNA	526	94.3	Nil
PL1	PCR product of LSU rRNA	595	95.5	One *Hind*III site
PL2	PCR product of LSU rRNA	595	93.8	One *Hind*III site
PL3	PCR product of LSU rRNA	735	88.8	One *Hind*III site
PLS	PCR product of tRNAs between LSU and SSU rRNA	973	87.3	Nil
PPH	PCR product of tRNAs before the repeat	1000	78.0	One *Eco*RI site
PLH	PCR product from LSU rRNA to His-tRNA	1118	82.3	Nil
PL470	PCR product from LSU to ORF470	1125	83.0	Nil
PRB	PCR product of the RpoB gene	516	87.9	One *Eco*RI site
PWQ	PCR product of the Phe-tRNA	161	69.6	Nil
PB-1	Sequence derived from clones spanning the Ile-tRNA, the ssu-rRNA, the lsu-rRNA and the ORF-470 genes	5849	88.5	4 *Hind III* sites
PB-2	Sequence derives from clones spanning the regions within the lsu-rRNA, the rps 4 and the cluster of 10 tRNA genes	2621	80.2	1 *Hind III* site and 1 *Eco RI* site

Table 2. Southern analysis of restriction digests.

Probe	*Hind*III digest	*Hind*III/*Eco*RI digest	*Eco*RI digest
PS1	H2	H2	N.D.
PLS	H4	H4	N.D.
PWQ	H6	E2	E2
PL1	H6	HE2	N.D.
PL2	H6	HE1, HE2	N.D.
PL3	H2, H4	H2, H4	E3
PRB	H6	HE2	E2, E3
PL470	H6	HE2	E3
PPH	H6	HE1	E3
PB6K-4	H3, H5	H3, H5	N.D.

PL2 and PL3 PCR products. In addition, both the H5 and the H3 fragments hybridised with probes corresponding to the *P.berghei* 6 kb mitochondrial DNA. This was not unexpected as the preparation was found to contain linear molecules as shown by electron microscopy (Fig. 1).

EXAMPLE 5

Physical and genetic map of the *P.berghei* 35 kb circle

A map of the approximately 35 kb *P. berghei* extrachromosomal circle was constructed based on the information from the restriction digests, Southern hybridisation experiments and the sequence analysis of the PCR fragments (Figure 4a). The PPH and PRB fragments each contained an *EcoR* I restriction site (Table 1). The three *EcoR* I and six *Hind* III fragments were arranged according to their hybridisation patterns. The PRB probe hybridised to both the E2 and E3 fragments from the *EcoR* I digest indicating that E2 is positioned next to E3 (Table 2).

The HE2 fragment obtained from the double digest with *EcoR* I and *Hind* III, hybridised to probes PRB, PL470 and PL2 while H2b and H4 hybridised to probe PL3. Both the PL2 and PL3 fragments are regions within the *lsu-rRNA* gene, whereas the PL470 fragment contains 3' end of the *lsu-rRNA* gene. Thus, the ORF470 must be located next to the PL2 fragment. H2b is situated between HE2 and H4 since H4 also hybridised with the PLS probe which contains the 5' ends of both the small and large subunits rRNA genes. H2a hybridised with probe PS 1 which corresponds to a region within the ssu-rRNA gene, therefore H2a must be located next to H4. Finally, HE1 is placed next to E1 as HE1 hybridised to probes PPH, PLH and PL2.

The arrangement of genes on the *P. berghei* circle spanning the *rpo B* gene and the cluster of 10 *tRNAs* genes is thus very similar to that of the *P.falciparum* 35 kb circle (Figure 4b). The *P.berghei* circle encodes organelle-like rRNAs, tRNAs, ribosomal proteins and RNA polymerase subunits, similar to those identified for *P. falciparum* (Preiser *et al.*, 1995).

EXAMPLE 6

Reverse transcription-PCR of LSU-rRNA and SSU-rRNA

In order to determine if the approximately 35 kb *Plasmodium berghei* extrachromosomal genetic element is transcriptionally active, total RNA from *P. berghei* was isolated using the RNeasy total RNA kit (Qiagen Inc., Chatsworth, CA, USA) and a combined reverse transcription - PCR (RT-PCR) reaction was carried out to amplify *lsu-rRNA* or *ssu-rRNA* transcripts.

Total RNA was isolated from *Plasmodium berghei* using the Qiagen RNeasy Total RNA kit (Qiagen Inc., Chatsworth, CA, USA). Parasites from 10 infected mice with a parasitemia of 60% were resuspended in 350 μl of lysis buffer RLT and homogenised using a QIAshredder (Qiagen Inc.). The homogenate was cleared of insoluble material by centrifugation and 1 volume of 70% ethanol was added. The entire sample was then added to the RNeasy spin column and washed with RW1 buffer followed by two washes with RPE buffer. The RNA was eluted out with 35 μl of water. 5 μl of the RNA was used as starting material for the Access RT-PCR system (Promega, Madison, USA). Two primer sets, L/L (SEQ ID Nos: 5 and 6) and S/S (SEQ ID Nos: 9 and 10) were used. The manufacturer's protocol was followed with the exception of the annealing step for PCR amplification. Annealing was allowed to proceed at 55°C for 1 minute. The PCR products were separated on a 1% (w/v) agarose gel and visualised by ethidium bromide staining.

Amplification using the RT- PCR kit from Promega and a set of primers homologous to the ssu-rRNA produced a 526 bp fragment while amplification using a set of *lsu-rRNA* specific primers resulted in a 594bp fragment (Fig. 5).

EXAMPLE 7

Assay of blood samples for the presence of *Plasmodium ssp.*

A total of 482 *Plasmodium*-infected blood samples from four different locations, Singapore, Laos, Pakistan, India and Colombia and a defined number of negative control

blood samples, were analysed for the presence of *Plasmodium* extrachromosomal genetic elements, using the polymerase chain reaction.

Briefly, 10-100μL of whole patient blood (either peripheral blood from a finger prick sample or venal blood) was spotted onto a filter disc or equivalent solid support and directly amplified, using each of the primer pairs:

L/L PRIMER PAIR:

SEQ ID NO:5:	5'-GACCTGCATGAAAGATG-3'
SEQ ID NO:6:	5'-GTATCGCTTTAATAGGCG-3'

L/S PRIMER PAIR:

SEQ ID NO:7:	5'-GCCACTACTATGAAAATC-3'
SEQ ID NO:8:	5'-GCGTTCATTCTGAGCTAG-3'

S/S PRIMER PAIR:

SEQ ID NO:9:	5'-GCGGTAATACAGAAAATGCAAGCG-3'
SEQ ID NO:10:	5'-AGCACGAACTGACGACAGCCATGCAC-3'

PCR Buffer used in the amplification reactions comprised the following:

70mM Tris. pH8.8

20mM Ammonium sulphate

1 mM DTT

0.1μg/μL BSA (or 0.01% geletin)

2.5mM $MgCl_2$

Each 100 μL reaction included 0.4μg of each primer, 0.8mM dNTP mixture and 5U of *Taq*I polymerase.

The template DNA was fixed with methanol for 5 mins. A "hot start" was carried out at 95°C for 5 minutes without the dNTPs and Taq polymerase. This was followed by the addition of

dNTPs and Taq polymerase and 40 cycles of denaturation (90°C, 1.0 mins), annealing (56°C, 2 mins) and extension (72°C, 1 min). The PCR products were analysed by agarose gel electrophoresis.

The results are shown in Tables 3 and 4. The L/L primer set was capable of identifying *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae* in 100% of cases, suggesting that this primer pair is useful in the genera-specific diagnosis of *Plasmodium* infection. The S/S primer set was capable of efficiently diagnosing *P. falciparum* and *P. malariae* in 100% of cases. In marked contrast, the L/S primer set resulted in only poor diagnosis of *P. vivax* and *P. malariae*, however detected the presence of *P. falciparum* in blood samples, suggesting that this primer pair is species-specific. The human actin primer set AC1/2 were used as positive controls.

Results also indicate that the selection of primer pairs in the diagnostic assay was of primary importance in determining the reliability of the assay in diagnosing infection by *Plasmodium ssp.*

TABLE 3

Number and origin of *Plasmodium* infected samples

Origin	Number	P.fal	P.viv	P.mal	P.ova	Mixed	Controls
Singapore	74	15	26	2	2	3	26
Laos	16	15	1	-	-	-	-
Pakistan	68	14	53	-	-	-	1
India	11	1	10	-	-	-	-
Colombia	313	1	29	-	-	-	283
Total	482	46	119	2	2	3	310

TABLE 4

PCR results using the primer pairs L/L, L/S, S/S and AC1/2

Species	L/L	L/S	S/S	AC1/2
P.fal	46/46 (100%)	14/20 (70%)	21/21 (100%)	nd
P.viv	119/119 (100%)	6/58 (10%)	48/57 (84%)	nd
P.mal	2/2 (100%)	0/1 (0%)	1/1 (100%)	nd
P.ova	2/2 (100%)	nd	nd	nd
mixed	3/3 (100%)	nd	nd	nd
controls	0/310 (0%)	nd	nd	92/92 (100%)

nd = not done

EXAMPLE 8

Direct PCR amplification of extrachromosomal *Plasmodium* DNA from dried blood spots

1. *Specimen Collection*

Blood was collected by fingerprick (5-10 μl) or by venipuncture from subjects with Giemsa smear-positive *Plasmodium falciparum*, *Plasmodium vivax* and *Plasmodium malariae* malaria as well as from healthy controls, and spotted in replicates onto Whatman filter paper. *Plasmodium berghei* (ANKA) infected mouse blood (5μl) was collected from the tail. *Plasmodium berghei* infections were maintained by serial blood passage of 10^7 parasites. Dried blood spots were placed individually into 200μl PCR tubes and fixed with the addition of methanol for 5 minutes. The methanol was poured off and the blood spot was dried thoroughly prior to PCR amplification.

2. *PCR amplification*

Amplification was carried out as previously described (Long *et al*, 1995) with some modifications. Each 100μl reaction mixture contained 1xPCR buffer (70 mM Tris, pH 8.8, 20 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.1 μg/μl BSA) 2.5 mM $MgCl_2$, 0.4μg of each primer, 5

units of Taq DNA polymerase (Amersham) and 0.2 mM of each dNTPs. Reaction tubes were overlaid with one drop of mineral oil. The reaction was soaked at 95°C for 5 minutes then held at 80°C prior to the addition of Taq DNA polymerase and dNTPs. Amplification involved 40 cycles of 1 minute denaturation at 90°C, 2 minute annealing at 52°C and 3 minutes primer extension at 72°C. A 5 minute primer extension at 72°C was included following the final cycle.

3. *Sequences of primers.*

The primers used for amplifying the LSU-rRNA gene were as follows:

L1 5' GAC CTG CAT GAA AGA TG 3' (SEQ ID NO: 5); and

L2 5'GTA TCG CTT TAA TAG GCG 3' (SEQ ID NO: 6).

A second set of primers were designed to amplify the dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene from *P.berghei* genomic DNA in control experiments:

DHFR1 5' GCA ATA TGT GCA TGT TGT AAA 3'; and (SEQ ID NO:48)

DHFR2 5'ATT CTT TAT AAA CAG ACG 3' (SEQ ID NO:49)

The primers used for amplifying the human β-actin gene were as follows:

AC1 5' GGG CGA CGA GGC CCA GAG C 3'; (SEQ ID NO:50)

AC2 5' GCA TCC TGT CGG CAA TGC C 3'; (SEQ ID NO:51)

AC3 5' AAG GAG AAG CTG TGC TAC 3'; and (SEQ ID NO:52)

AC4 5' TCA TGA TGG AGT TGA AG 3' (SEQ ID NO:53)

4. *Agarose gel electrophoresis*

10 μl of each PCR product was resolved in 1% agarose gels with TAE electrophoresis buffer (40mM Tris-acetate and 1mM EDTA, pH 8.0). Electrophoresis was carried out at 100V for 1.5 hours and the fragments were visualized under UV.

5. *DNA sequencing protocol*

The PCR products were loaded onto a 1% (w/v) low-melting point agarose gel and extracted

by the freeze-thaw method (Shoemaker and Salyers, 1990). They were then cloned into the pGEM-T vector (Promega). The clones were sequenced using the ABI PRISM Dye terminator cycle sequencing kit (Perkin Elmer) on the 373 DNA sequencer from Applied Biosystems. Multiple sequence alignment using the cluster method was carried out with the DNASTAR programme.

6. *Results*

6.1 *Detection of P.berghei infection in blood spots*

Conditions for the PCR amplification of *P.berghei* infected mouse blood spotted on filter paper were optimised using DHFR1 and DHFR2 primers. Once these conditions were established, the sensitivity of the LSU-rRNA primer set was compared with that of the DHFR-TS primer set. The LSU-rRNA primer set was designed to amplify a 594bp fragment from the *P.berghei* circular DNA while the DHFR-TS primer set amplified a 511bp fragment from *P.berghei* genomic DNA. Blood spots were prepared daily for 5 days from a mouse which was initially infected with 5 x 10^4 parasites. Giemsa staining of thin blood films from the same animal was done daily. The LSU-rRNA primer set was more sensitive than the DHFR-TS primer set in detecting parasite DNA. The amplified LSU-rRNA fragment was detectable by ethidium bromide staining one day after infection (Figure 6a) while the DHFR-TS PCR product was only visible two days post-infection (Figure 6b). At these two time points, no parasite was detected on the corresponding Giemsa-stained blood films. Parasites were only observed on the film three days post-infection.

6.2 *PCR amplification of blood spots from malaria infected patients.*

The above PCR amplification protocol was also applied to blood spots from 31 malaria-infected patients. 15 of these samples were obtained from patients admitted to the National University Hospital in Singapore. Of these, 7 had *P.falciparum* infection, 1 had *P.malariae* and the remaining had *P.vivax* as determined by Giemsa and Quantitative Buffy Coat (QBC) diagnosis. All samples were positive for amplification with LSU primers (data not shown). The other 16 samples were from patients in Laos with 15 *P.falciparum* infections and one *P.vivax* malaria infection as determined by Giemsa diagnosis. LSU-rRNA PCR

amplifications were positive for all 16 specimens. As shown in Figure 7, the PCR products from 11 of the 16 Laotian specimens. Eight healthy persons and total DNA from two human carcinoma cell lines, CaSki and HeLa were used as controls. These were all negative when using the LSU-rRNA primer set for PCR amplification but were all positive for human β-actin (Figure 8, compare panels a and b).

EXAMPLE 9

Sequence alignment of LSU-rRNA extrachromosomal DNA from various *Plasmodium* species

The LSU-rRNA fragments amplified from the blood spots as described in Example 8 were cloned into the pGEM-T vector and sequenced. In addition to amplified products from the Singaporean and Laotian patients, we also amplified and sequenced LSU-rRNA fragments from Indian, Colombian and Pakistani patients. The published *P.falciparum* sequence (C10 strain) was used as the basis for all alignments and comparisons.

Comparison of the *Plasmodium* species used in this study showed that this region of the LSU-rRNA gene is highly conserved and the similarity between *P.falciparum*, *P.vivax*, *P.malariae*, *P. ovale* and *P.berghei* is greater than 91% (Table 5). The similarity between the C10 and other *P.falciparum* sequences ranged from 98.3%-99.8%, while that between the C10 and the *P.vivax* sequences ranged from 91.1- 99.7%. The greatest divergence in sequence was observed from the *P.vivax* specimens from Pakistan and Colombia. In all cases, divergence in sequence was due to 1 or 2 base changes in isolated regions within the LSU-rRNA fragment (Figure 9A-9J)

TABLE 5

Percent homology of LSU-rRNA sequences with *P.falciparum* (C10 strain) sequence

Name of sequence[1]	Similarity Index to Pf(C10)[2]
Pf10/P	98.3
Pf11/P	98.5
Pf19/I	99.7
Pf20/L	99.7
Pf18/S	99.8
Pv12/P	93.4
Pv13/P	92.9
Pv15/I	99.5
Pv16/L	99.7
Pv17/S	93.4
Pv86/C	91.1
Pm1/S	93.2
Pm38/S	92.9
Po35/S	93.4
Po36/S	93.2
Pb(ANKA)	94.2

[1] Pf denotes *P.falciparum*, Pv denotes *P.vivax*, Pm denotes *P.malariae* and Pb denotes *P.berghei*. The alphabet at the end of each name indicates the origin of the specimen; P=Pakistan, I=India, L-Laos, C=Colombian and S=Singapore. The GenBank accession numbers for Pf(C10) and Pb(ANKA) are X95275 and U79731 respectively.

[2] Similarity index obtained using the Martinez-Needleman-Wunsch DNA alignment programme.

EXAMPLE 10

Discussion

In this study, we have shown that it is possible to amplify the extrachromosomal circular plastid-like DNA found in *Plasmodium ssp.* This has allowed us to proceed with characterising the LSU-rRNA gene from the circular DNA of malaria-infected patients using only a small volume of blood spotted on filter paper.

We have designed a pair of primers based on the sequences from *P.falciparum* and *P.berghei* such that the primers are completely homologous for both species. Using these primers, we have been able to amplify the corresponding LSU-rRNA fragment from *P.falciparum*, *P.vivax*, *P.malariae* and *P.berghei* infected blood. Sequence analysis of these fragments indicates that this region of the LSU-rRNA is highly conserved between different species of *Plasmodium*. In addition, different geographic isolates of *P.falciparum* and *P.vivax* from Asia do not show distinct variations for the LSU-rRNA fragment. GenBank searches indicate that this fragment sequence is unique.

The high homology between the various *Plasmodium* species has led us to examine if the LSU-rRNA specific primers are useful for the detection of malaria infections. Using *P.berghei*, the LSU-rRNA primer set was shown to be more sensitive than the DHFR primer set in parasite detection in mouse blood spots. All 31 patient blood spots tested were positive regardless of the *Plasmodium* species involved while none from healthy persons was positive. These results indicate that the LSU-rRNA primers may be useful for the diagnosis of malaria infection.

The ease of direct PCR amplification of extrachromosomal *Plasmodium* circular DNA from dried blood spots has provided us with the means to study and characterise the genes present on this DNA molecule. To date, none of the genes on the circular DNA of *P.vivax* and *P.malariae* has been described. This is the first description of an analysis of the LSU-rRNA gene from different field isolates of *P. ovale, P.vivax, P.malariae* and *P.falciparum*. More investigations are being carried out to determine the extent of sequence conservation and

arrangement of the genes on the circular DNA from different *Plasmodium* species.

EXAMPLE 11

PCR amplification and sequence analysis of cox I gene

To obtain the complete sequence of the mitochondrial cox I gene, a set of primers was designed based on the published *P.falciparum* sequence (GenBank accession number M76611). PCR using this primer set with blood spots from *P.vivax* infected patients resulted in fragments of 1.5kb in size (Figure 10). These were cloned into pGEM-T vector (Promega). The clones were sequenced in both directions using the ABI PRISM dye terminator cycle sequencing kit on the 374 DNA sequencer from Applied Biosystems. DNA sequence alignments were carried out using the Martinez/Needleman-Wunsch DNA alignment. The DNA sequences from 4 different *P.vivax* isolates were highly conserved (greater than 99% similarity). However, these sequences were less homologous (83%) when compared with the corresponding cox I gene from *P.falciparum*.

EXAMPLE 12

Plasmodium species identification in blood samples

In order to differentiate between *P.vivax* and *P.falciparum* infection, two sets of *P.vivax* specific primers (PV1 - SEQ ID No: 12 and P2 - SEQ ID No: 11; PV2 - SEQ ID No: 13 and P3 - SEQ ID No. 14) and four sets of *P.falciparum* specific primers (PF1 - SEQ ID No: 15 and P2 - SEQ ID NO 11; PF2 - SEQ ID No: 16 and P2 - SEQ ID No: 11; PF3 - SEQ ID No: 17 and P2 - SEQ ID NO 11; PF2 - SEQ ID No: 16 and P4 - SEQ ID No: 18) were designed based on the mitochondrial cox I genes of the two species. PCR assays were carried out on whole patient's blood spotted onto a filter disc as described for Example 7. The PCR products were analysed by agarose gel electrophoresis.

The results are shown in Tables 6, 7, 8 and 9. The three sets of *P.falciparum* specific primers only detected *P.falciparum* infected blood but not the other three human *Plasmodium* species. The *P.vivax* specific primers (PV1/P2) detected only 92% of the *P.vivax* infected blood and a

false positive with P.malariae was also observed. This primer set does not react with *P.falciparum* or *P.ovale* (Table 6).

TABLE 6

Results using Plasmodium species specific primer set PF1/P2 and PV1/P2

Species	*P.falciparum* primer set I (PF1/P2)	*P.vivax* primer set I (PV1/P2)
P.fal	12/12	0/12
P.viv	0/26	24/26
P.mal	0/2	1/2
P.ova	0/2	0/2
Pm/Pf	1/1	0/1
Pv/Pf	2/2	2/2
Controls	0/7	0/7

TABLE 7

Results using Plasmodium species specific primer set PF2/P2

Species	*P.falciparum* primer set II (PF2/P2)
P.fal	6/6
P.viv	0/14
P.mal	0/2
P.ova	0/2
Controls	0/7

TABLE 8

Results using Plasmodium species specific primer set PF3/P2

Species	*P.falciparum* primer set III (PF3/P2)
P.fal	9/9
P.viv	0/12
P.mal	0/2
P.ova	0/2
Controls	0/7

TABLE 9

Results using Plasmodium species specific primer sets PF2/P4 and PV2/P3

Species	*P.falciparum* primer set IV (PF2/P4)	*P.vivax* primer set II (PV2/P3)
P.fal	20/20	0/20
P.viv	0/31	31/31
Pv/Pf	3/3	3/3
Pm/Pf	2/2	0/2
Pv/Po	0/3	3/3
Controls	0/10	0/10

EXAMPLE 13

Sensitivity of PCR assay for Plasmodium species in blood samples

The minimum number of parasites detectable by PCR assay was determined by using 1µl of whole patient blood or diluted blood spotted on filter disc. Using the L/L (SEQ ID NOS 5 and 6), the minimum number of parasite detected is 4 (Figure 11).

EQUIVALENTS

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. The invention also includes all of the steps, features, composition and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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